Studies on Microbial Quantity and Dissolved Oxygen Content of Raw Chilled Milk Samples Based On Methylene Blue Reduction Test and Oxidation Reduction Potential

M.Baby Jenitta, J.Sherly., K.Mohan

***Abstract*— Ten raw milk samples, from various districts of Tamilnadu were analysed to determine their oxygen content for grading them before use. The results and time taken for both Methylene Blue Reduction Test (MBRT) and the alternate method such as Oxidation Reduction Potential (ORP) and Dissolved Oxygen (DO) are compared and analysed. Standard plate count are analysed to check the accuracy of the result. For all samples the ORP, MBRT and DO where checked and also plated for SPC and coli count were checked. The results for ORP were found to parallel with MBRT. Standard Plate Count increased with decreasing values of ORP and MBRT. The time taken for ORP was nearly within 10minutes and that for MBRT is by one hour or more. Therefore the ORP method developed by us is a suitable and alternate rapid test to access the MBRT of the raw chilled milk in the dairy industry. The value for ORP ranges between 27 and 146for MBRT values between 15 to 85 minutes. The milk sample is of good quality for ORP values greater than 40 and excellent for ORP values greater than 70. If the SPC is less than 2,00,000 then the quality of the milk is very good and it is good when SPC lies between 1 to 5 million and it is poor above this value.**

***Index Terms*— MBRT; ORP; DO; SPC; Coliform count.**

I. INTRODUCTION

Milk of cow's has long been considered a highly nutritious and valuable human food, and is consumed by million daily in a variety of different products. Milk is compulsory part of daily diet for the expectant mothers as well as growing children (Javaid et al., 2009). Milk being nutritious food for human beings, also serves as a good medium for the growth of many microorganisms, especially Lactobacillus, Streptococcus, Staphylococcus and Micrococcus sp. Raw milk, as it leaves the udders of healthy animals normally contains very low numbers of microorganism. Bacteria related to food borne illness are destroyed by proper pasteurization. In India raw milk is traditionally consumed at the small farms where it is produced or fermented into different products. A number of bacteria including Staphylococcus aureus, Escherichia coli and Salmonella have been recovered from raw milk (De Buyser et al., 2001) and some of these have been determined to be pathogenic and toxicogenic, and implicated in milk- born gastroenteritis (De Buyser et al., 2001). Raw milk is subjected to various quality tests when arrived at processing plants such as 5fat, solid not fat (SNF), % Acidity, specific gravity etc. These types of physical and chemical test are common and routinely conducted to classify the milk into quality grades for pricing purpose.

Methylene Blue Dye Reduction Test (MBRT) was used in evaluating cell viability (Nandy et al., 2010). The methodology employed the enzymatic reduction of methylene blue by a metabolically active organism turning the Methylene Blue colorless. The disappearance of the color is due to the removal of oxygen from milk and formation of reducing substances during bacterial metabolism (Impert et al., 2002). Methylene Blue (MB) dye has been employed to check for the overall microbial load and quality control of milk and other liquid foods (Impert et al., 2002).

Attempts have been made to assess microbial quality by measuring dissolved oxygen (DO) in milk sample and its reduction over time. The idea was to relate the rate of change in (DO) levels with the population of bacteria in the sample (Homhual et al., 2001). In the present studies ORP method is used as an alternate method to MBRT method to check for the overall microbial load and quality control of milk and grade the quality of raw milk before processing them in the processing plants. The results for ORP were found to parallel with that of MBRT. The time taken for ORP was nearly within 10minutes and that for MBRT is by one hour or more. Standard plate count and coliform count were analysed to check the accuracy of the result their values were compared in graphs and are found to decrease with the values of ORP and MBRT. Thus ORP method developed by us is a suitable alternate and a rapid test to access the MBRT of the raw chilled milk in the dairy industry.

Coliform bacteria have minimum generation time (Muhammad et al., 2009), and multiply at a rapid rate to reach its number to un-hygienic levels. (Asmahan et al., 2011) performed a coliform test by plating one ml sample onto MacConkey agar media. The plates were incubated at 37ºC for 48 h and the counts were presented as colony forming unites per gram (cfu/g). plates showing positive coliform were subjected to the confirmatory test using Brilliant green bile lactose broth in test tubes with inverted Durham tubes and incubated at 44ºC for 48 h. Each positive tube was sub cultered into broth medium and then incubated at 44.5ºC for 24 h. Tubes showing gas productions were considered E.coli positive. All the samples positive for E.coli contamination were confirmed using Gram's staining, cultural and biochemical examinations. For the isolation and identification

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of E.coli, the enriched sample was cultured on selective medium Levine Eosin Methylene Blue (EMB) Agar and incubated at 37ºC for 24 h.

In order to assess microbial quality of raw milk Standard plate Count (SPC) is recommended. SPC requires at least 48 hours to classify milk into quality grades such as if CFU/ml $<2x10^5$ the milk sample will be graded as 'excellent' , however, if the sample contain $>5x10^6$ CFU/ml of milk the milk sample would be 'bad' (Impert et al., 2002). Several studies have proved a strong correlation (R2 0.81-0.89) of MBRT method with SPC (Homhual, 2000,). However, a good milk sample would take 8-10 hours to yield results which are still a relatively longer time from operational point of view (Imran et al., 2010).Aim is to reduce the time taken to analyze the quality of raw milk and to plate the samples for E.coli and SPC. Objectives are to collect the raw milk samples, to perform Methylene Blue Reduction Test, to perform Oxidation Reduction Test, tO perform Standard Plate Count to check the accuracy of results, to perform and analyse the results.

II. MATERIALS AND METHODS:

A. METHYLENE BLUE REDUCTION TEST (MBRT)

Methylene blue reduction test reflects the bacterial load in milk and it is indicated by time taken for methylene blue dye in milk to change in color from blue to white. It is a traditional method which is followed throughout the globe.Take 10ml of milk sample in a sterile test tube. Add 1ml of methylene blue dye to the sample and close the test tube with rubber bungs. Mix the contents of the test tubes thoroughly by inverting it once or twice. The test tubes were placed in water bath at 37ºC. The test tubes were checked for decolourisation after every 5 minutes until there is a complete disappearance of the blue colour. Results were tabulated and the time taken for complete decolourisation expresses the quality of the microbial milk samples.

Fig 1. Methylene Blue Reduction Test

B. OXIDATION REDUCTION POTENTIAL (ORP):

It is a measurement to oxidize the contaminants. It is a knowledge of the relation of species, and the number of the organisms in milk, to the oxidation- reduction potential. This might be the value in interpretation of results, with milks in which these organisms predominate.

C. METHOD:

The ORP meter measures very small voltages generated in the raw sample. The electrode is made of platinum or gold which reversibly looses its electrons to the oxidizer. A voltage is generated which is compared to the silver (reference) electrode in silver salt solution similar to pH electrode. The more oxidizer available, the greater the voltage difference between the solution.

Fig 2. ORP Meter

a) DISSOLVED OXYGEN (DO):

The clark type electrode is the most used oxygen sensor for measuring oxygen dissolved in a liquid. The basic principle is that there is a cathode and an anode submerged in an electrolyte. Oxygen enters the sensor through a permeable membrane by diffusion, and is reduced at the cathode, creating a measurable electrical current. It is possible to measure oxygen in the sample. There is linear relationship between the oxygen concentration and the electrical current. It is possible to measure oxygen in the sample.

Fig 3. Dissolved OXYGEN Probe

b) COLIFORM COUNT:

The members of the coliform group of bacteria eg. Escherichia are commonly found in dairy products. As these organisms are capable of growing rapidly at room temperature (30-45ºC) and produce acid, gas and objectionable taints in the products they are considered to be very undesirable contaminants. The estimation of coliform bacteria in milk is, therefore, very important in quality control work. The test for coliform organisms based on the principle

that the members of this group are capable of producing acid and gas from lactose in the presence of bile salt. A small amount of milk (1.0, 0.1 or 0.01ml) is added to liquid or solid media containing lactose and bile salt with a suitable indicator. Production of acid and gas in liquid media and appearance of typical colonies of coliform on the plates is taken as evidence of coliform contamination. Transfer 1ml of the well mixed to 9ml of saline dilution blank and mix well by using cyclomixer. Transfer 1ml from first tube into a second tube of 9ml saline. Transfer 1ml from this suspension to a third tube of saline and continue the transfer to the fourth, fifth, sixth tubes of saline. Mark the dilution tubes as 1, 2, 3, 4. Inoculate 1ml proportions of the required dilution into sterile petri plates. Add 10-15ml of violet red bile agar to each plate previously melted and cooled to 45ºC. Mix the contents thoroughly and allow the agar to solidify. Invert and incubation for 24 hours, remove the plates and for typical colonies of coliform bacteria.

c) STANDARD PLATE COUNT (SPC):

The standard plate count or pour plate method is used for estimating the viable microorganisms in milk and milk products. The various factors which affect SPC include temperature of incubation, period of incubation, composition of plating medium, existence of bacterial clumps etc.,Transfer 1ml of the well mixed to 9ml of saline dilution blank and mix well by using cyclomixer. Transfer 1ml from first tube into a second tube of 9ml saline. Transfer 1ml from this suspension to a third tube of saline and continue the transfer to the fourth, fifth, sixth tubes of saline. Mark the dilution tubes as 1, 2, 3, 4. Inoculate 1ml proportions of the required dilution into sterile petri plates. Add 10-15ml of Trypton Glucose Yeast Extract Agar to each plate previously melted and cooled to 45ºC. Mix the contents thoroughly and allow the agar to solidify. Invert and incubation for 24 hours, remove the plates and for typical colonies of mesophilic organism. After incubation, bacterial cells grow in to distinct and isolated colonies which can be counted with the help of a colony counter. In order to calculate the total number of viable bacteria per g or ml of the sample, the number of the colonies developed on each plates are multiplied by the dilution factor.

III. RESULTS AND DISCUSSION

The raw milk samples were collected and are analysed for their MBRT and ORP values, SPC count, coliform count and their DO content were also analysed. The results obtained are given below

A. RESULT AND DISCUSSION FOR MBRT

Time required for reduction (hrs)	Grade / Quality of milk
5 and above	Very Good
3 and 4	Good
1 and 2	Fair
0.5 and below	Poor

Table 1. Result for MBRT

If the MBR time is by 5 to 6 hours the quality of the raw milk is excellent and it is good when the MBR time is between 3 to 4 hours. It is fair when the time ranges between 1 to 2 hours and is poor below this value is shown in table 1. Several studies have proved a strong correlation (R2 0.81-0.89) of MBRT method with SPC (Homhual, 2000, Ahmad, 2001). However, a good milk sample would take 8 to 10 hours to yield results which are still a relatively longer time from operational point of view (Imran et al., 2010). In raw milk sample, there was very low correlation (r=0.081) between total viable count and MBRT (Benson, 2002) and is shown in table 1.

B. RESULT FOR COLIFORM COUNT

The presence of dark red colonies measuring atleast 0.5mm in diameter constitutes a positive test shown in Fig 4. The coliform limits in the raw milk accepted internationally are > 100 cell/ml. (Salman and Hamad, 2011) have reported seasonal and geographical variation of coliform in milk samples.

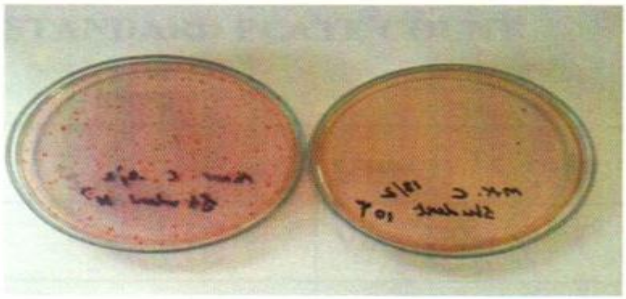


Fig 4. Result for coliform count

C. RESULT FOR STANDARD PLATE COUNT

The presence of white colour colonies indicates the presence of mesophilic organisms Fig 5. SPC requires atleast 48 hours to classify milk into quality grades such as if CFU/ml < 2x105 the milk sample will be graded as 'excellent', however, if the sample contain > 5x106 CFU/ml of the milk sample would be 'bad' (Imran et al., 2010).

Fig 5. Result for SPC

D. OBSERVATION FOR STANDARD PLATE COUNT

Count / ml	Quality / Grade
Less than 2,00,000	Very good
2,00,000	Good
1 – 5 million	Fair
Over 5 million	Fair

Table 2. Observation for SPC

If the SPC is less than 2,00,000 then the quality of milk is very good and it is good when SPC lies between 2,00,000 to 1 million. It is fair when the count lies between 1 to 5 million and it is poor above this value.

IV. COMPARISSON BETWEEN MBRT AND ORP

Sample	ORP	MBRT	Table MBRT	DO	Coli x 10^4	SPC x 10^6
Namakkal (S1)	27	15	18	0.30	520	285
Nainarpalayam (S2)	40	25	26.7	1.28	272	210
Athur (S3)	60	35	36	1.33	160	63
Mangalore (S4)	69	40	41	1.44	118	60
Siruvangore (S5)	75	45	45	1.47	110	52
Sanoor (S6)	93	50	45.8	1.47	22	17
China Selam (S7)	118	70	70	1.68	4	10
Sreevilliputhur (S8)	127	75	76.2	1.33	1	5
Krishnagiri (S9)	136	80	81.6	1.99	Ab	3
Manargudi (S10)	146	85	87	2.35	Ab	1

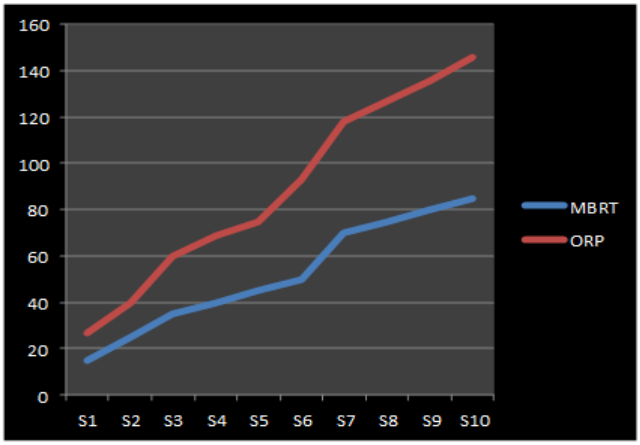
Table 3. Comparison between MBRT and ORP

Ten raw milk samples (s1, s2…s10), from various districts of Tamil Nadu were analysed and to determine their oxygen content for grading them before use. Table 4. is prepared to compare the values obtained by MBRT method ORP method, table or standard MBRT values, DO, COLI and SPC. The values obtained by MBRT and ORP were found to be linear hence showing that ORP method can be used as an alternate to MBRT method. The values are compared by graphs inorder to expel their relationship.

The MBRT depends upon the ability f bacteria in milk to grow and to consume the DO, which reduces the oxidation reduction potentials in the medium. (Srujana et al., 2011). SPC requires atleast 48 hours to classify milk into quality gades such as if CFU/ml <2x105 the milk sample will be graded as ‘excellent’, however, if the sample contain >5x106 CFU/ml of milk sample would be ‘bad’ (Imran et al., 2010). Thus the values are compared in table 3.

V. SAMPLE VERSES MBRT AND ORP

The values obtained by MBRT method and ORP method are plotted against the sample used inorder to compare them in graph1. The values were found to be linear with each other indicating same curves. The values increases and decreases at same points. MBRT was used in evaluating cell viability. The methodology employed the enzymatic reduction of methylene blue reduction by a metabolically active organism turning the Methylene Blue colourless, the rate of decoloration by the metabolically active cells can be coreelated to the number of viable cells, for this purpose, the slope of the MB decolration rate was calibrated with respect to colony forming units (CFU) obtained through plating and this method is successfully employed to characterize the viability of E.coli and B.subtilis. Further, the methodology was used to characterize the cannibalistic tendency of B.subtilis under nutritional limiting conditions. These studies revealed that MBRT can be successfully employed to quantify viable cell count in a duration of 30 minutes to 3 hours (Subir et al., 2010). MBRT is used to evaluate CFU of an aerobic microorganism. The disappearance of the color is due to the removal of oxygen from milk and formation of reducing substances during bacterial metabolism. Thus MBR has a close relation with ORP in milk samples and is shown in Graph 1.

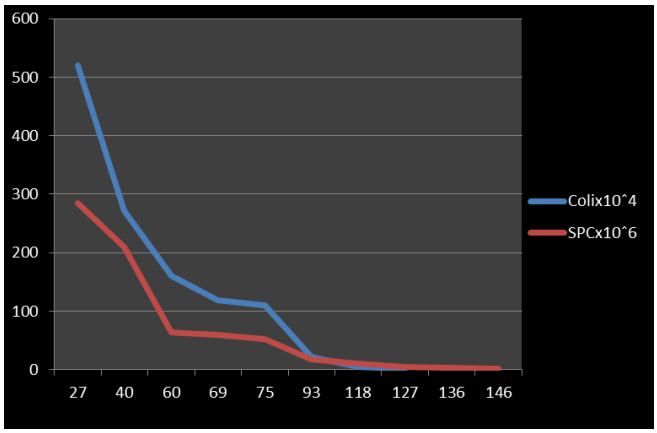


Graph 1.Sample Vs MBRT and ORP

A. ORP VERSES COLIFORM AND STANDARD PLATE COUNT

The colonies obtained for SPC and coli are plotted against the values obtained by ORP method used inorder to compare them in Graph 2. The values were found to be linear with each other indicating same curves. The values increases and decreases at same points. Both the curves have decreasing values with increase in ORP. The production of raw milk with SPC consistency of less than 10,000 cfu/ml is a reflection of good hygienic practices while an SPC more than 10,000 cfu/ml is reflection of poor hygienic practices during raw milk production. The coliform limits in the raw milk accepted internationally are > 100 cell/ml. Salman and Hamad (2011) have reported seasonal and geographical variation of coliform in milk samples. (Asmahan et al., 2011) performed a coliform test by plotting one ml sample onto MacConkey agar media. The plates were incubated at 37ºC for 48 h and the counts were presented as colony forming unites per gram (cfu/g).

Plates showing positive coliform were subjected to the confirmatory test using Brilliant green bile lactose broth in test tubes with inverted Durham tubes and incubated at 44ºC for 48 h. Tubes showing 'gas' productions were considered E.coli positive. All samples positive for E. coli contamination were confirmed using Gram's staining, cultural and biochemical examinations. For the isolation and identification of E.coli, the enriched sample was cultured on selective medium Levine Eosin Methylene Blue (EMB) Agar and incubated at 37ºC for 24 h. Hence there is a close relationship between coli and ORP as shown in Graph 2.



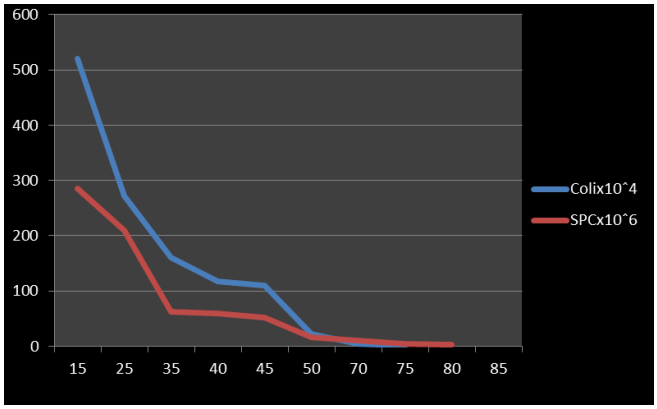
Graph 2. ORP Vs coliform and SPC

B. MBRT VERSES COLIFORM AND STANDARD PLATE COUNT:

The colonies obtained for SPC and coli are plotted against the values obtained by MBRT method used inorder to compare them in Graph 3. THE values were found to be linear with each other indicating same curves. The values increases and decreases at same points. Both the curves have decreasing values with increase in MBRT value. And is similar to those curves obtained in Graph 2.

MBRT was used in evaluating cell viability . The methodology employed the enzymatic reduction of Methylene Blue by a metabolically active organism turning the methylene blue colorless, the rate of deoloration by the metabolically active cells can be correlated to the number of viable cells, for this purpose, the slope of MB decoloration rate was calibrated with respect to colony forming units (CFU) obtained through plating and this method is successfully employed to characterize the viability of E.coli and B.subtilis.

In order to assess microbial quality of raw milk SPC is recommended, SPC involves sampling of raw milk, inoculation into growth media and incubated at favorable temperature that would allow growth of bacteria and the number of bacteria is counted in terms of colony forming units per ml of milk (CFU/ml). SPC requires at least 48 hours to classify milk into quality grades such as if CFU/ml < $2x10^5$ the milk sample will be graded as 'excellent', however , if the sample contain >$5x10^6$ CFU/ml of milk the sample would be 'bad' (Imran et al., 2010). Therefoe, this is not practical test to perform on arrival of milk lot to appreciate the price, milk industry relies on an indirect method of quality assessment; MBRT. In this method a blue dye is added to milk which over time becomes white due to metabolic activity of bacteria.



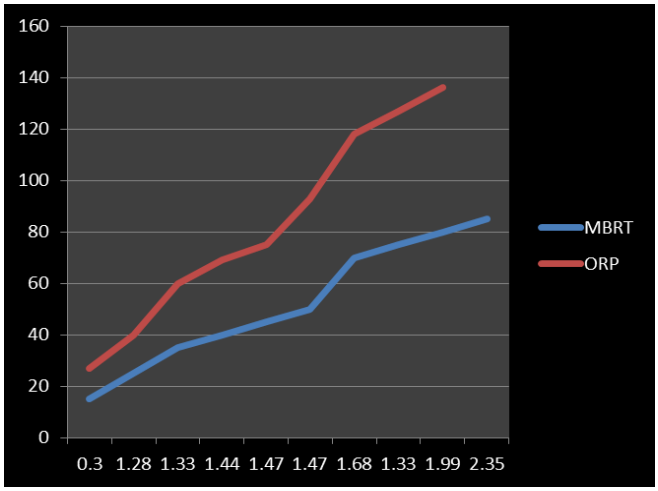
Graph 3. MBRT Vs coliform and SPC

If more bacteria are present in the sample, faster the metabolic activity will be which result into disappearance of dyeing the milk. Several studies have proved a strong correlation (R2 0.81-0.89) of MBRT method with SPC (Homhual, 2000.). Hence there is a close relationship between the bacteria in milk, SPC and MBRT and is shown in Graph 3.

C. DO VERSES MBRT AND ORP

The value obtained by MBRT method and ORP method are plotted against the value of DO inorder to compare them. The values were found to be linear with each other indicating same curves. The values increases and decreases at same points as shown in Graph 4.

Methylene Blue (MB) dye has been employed to check for the overall microbial load and quality control of milk and other liquid foods (Impert et al., 2002). Because of its size and positive charge, it does not enter into the cells appreciably. It gets reduced to 'leuko' or colorless form of MB at the cell surface via reductase enzymes present in the cell membrane. This colorless form of methylene blue is (MBH) I uncharged, lipophilic, and enters cells by diffusion across the plasma membrane where it is re-oxidized and thus sequestered within the cells (May et al., 2003). MBRT is used to evaluate CFU of an aerobic microorganism. The disappearance of the color is due to the removal of oxygen from milk and formation of reducing substances during bacterial metabolism. The MBRT depends upon the ability of bacteria in milk to grow and to consume the dissolved oxygen, which reduces the oxidation reduction potentials in the medium. (Srujana et al., 2011).And thus the relation between MBRT value is shown in Graph 4.



Graph 4. DO Vs MBRT and ORP

VI. CONCLUSION

This study has revealed a close relationship between Oxidation Reduction Potential and Methylene Blue Reduction Test to determine the oxygen content of raw milk for grading them before use. The results and time taken for both Methylene Blue Reduction Test (MBRT) and the alternate method such as Oxidation Reduction Potential (ORP) and Dissolved Oxygen (DO) are compared and analysed with tables and graphs. The results for ORP were found to parallel with MBRT. Standard Plate Count increased with decreasing values of ORP and MBRT. The value for ORP ranges between 27 and 146 for MBRT values between 15 to 85 minutes. The milk sample is of good quality for ORP values greater than 40 and excellent for ORP values greater than 70. If the SPC is less than 2,00,000 then the quality of the milk is very good and it is good when SPC is lies between 2,00,000 to 1 million. It is fair when the count lies between 1 to 5 million and it is poor above this value. The time taken for ORP was nearly within 10 minutes and that for MBRT is by one hour or more. Therefore the ORP method developed is a suitable and alternate rapid test to access the MBRT of the raw chilled milk in the dairy industry.

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